American Fern Journal 92(1):10–19 (2002)

Crystals Associated with the Intertracheid Pit Membrane of the Woody Fern *Botrychium multifidum*

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ABSTRACT.—CALCIUM-containing crystals have been found in the lumens of secondary tracheids in the rhizome of the woody fern *Botrychium multifidum*. These crystals are styloids with rough, pyramid- shaped ends. The crystals are usually single; however, conjoined or grouped crystals were also found. Crystal formation apparently has no constant relation to the pit membrane, but crystals of mature tracheids are often associated with the pit membrane or are located in the pit areas. Crystals were also located between the helical thickenings of the lumen walls. No crystal chamber or crystal sheath was found in association with the crystal body.

Crystals are a common feature in many plant tissues (Scurfield and Mitchell, 1973), and more than 1000 crystal producing woody plants, spanning 160 families, were described at the light microscopic level by Chattaway (1955, 1956). Scanning electron microscopy has allowed for more rapid identification of crystals in plant tissues and a clearer picture of their morphology (Scurfield and Mitchell, 1973). Although crystals in xylem tissue have been reported in the vessels of *Intsia* Thouars (Fabaceae; Hillis, 1996), *Torreya yunnanesis* C.Y. Cheng & L.K. Fu (Taxaceae; Kondo *et al.*, 1996), and *Polyalthia* Blume (Annonaceae; Scurfield and Mitchell, 1973), they are most commonly found in the xylem parenchyma, septate fibers, or vessel tyloses (Scurfield and Mitchell, 1973).

The formation of crystals by *Botrychium*, the only extant fern that produces wood (Gifford and Foster, 1989), has not been previously reported. During our studies of the torus-bearing pit membrane in the tracheid of *Botrychium multifidum* (S.G. Gmelin) Rupr., we discovered occasional instances of crystals associated with the pit membrane. This paper describes the morphology of these crystals as observed with SEM. The discoverey of these very small crystals was unexpected, and our exploration of them to this point has been strictly descriptive. However, in our discussion we explore several possible reasons for crystal formation in this wood.

MATERIALS AND METHODS

Rhizome samples of upright or orthotropous rhizomes of *Botrychium multifidum*. were collected by Dr. D. W. Stevenson (New York Botanical Garden, New York City, U.S.A.) from Plumas County, California and fixed in FPA. The collection site (elevation 2000 m) is rocky mountain soil at the edge of a meadow and the ground is frozen for much of the year. The samples were typical rhizomes selected as random samples and representative of the population. In

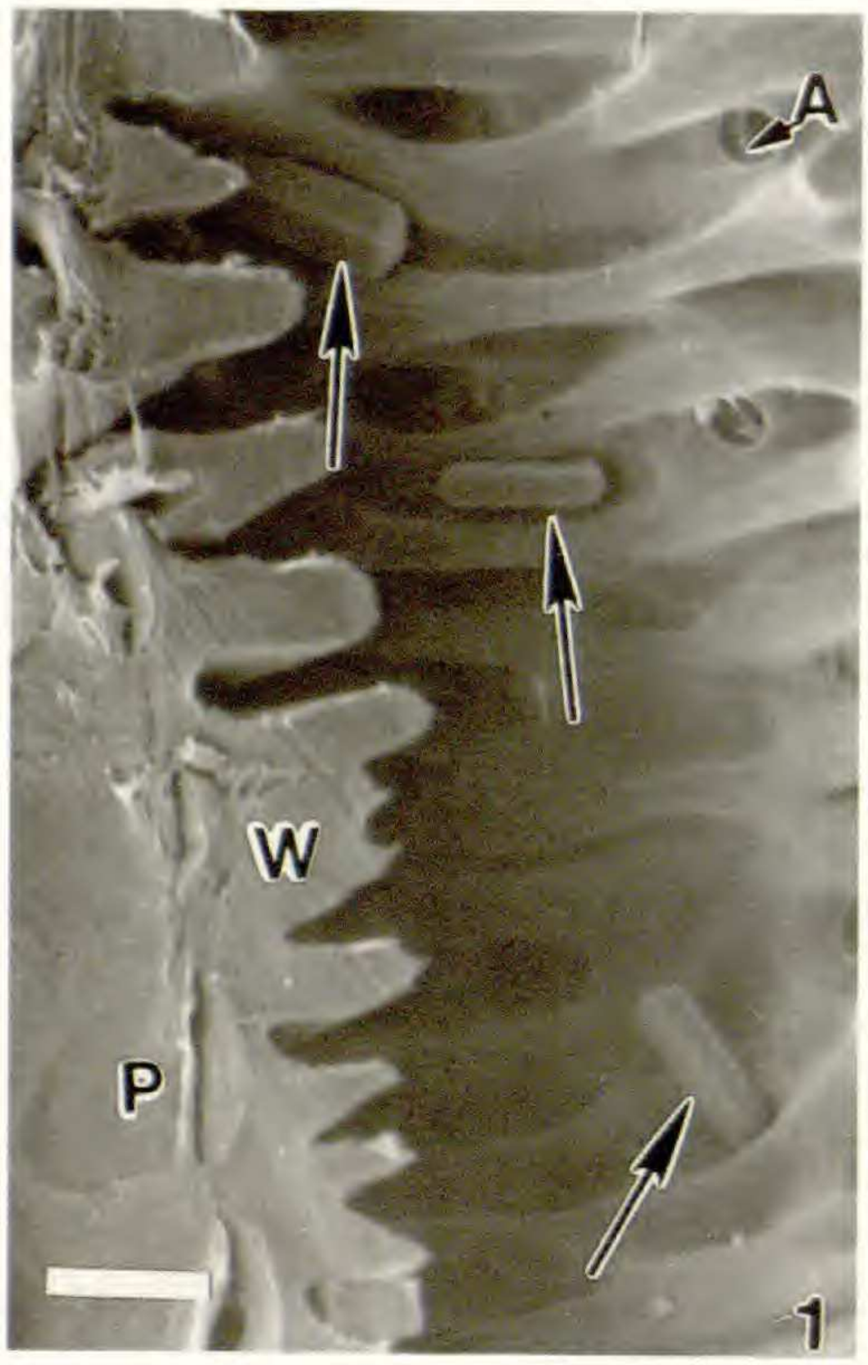
our lab, samples were cut transversely into 1–2 mm pieces that were placed into 50% ethanol and then dehydrated through a graded alcohol series. Samples then were cut into small wedges, placed into hexamethyldisilazane (HMDS) for 2 hours (Nation, 1983), and subsequently placed under a chemical hood overnight to dry. Dry samples were attached to aluminum stubs with double-sided sticky tape and coated with gold-palladium. For comparison purposes, samples of *Botrychium dissectum* Sprengel and *B. virginianum* (L.) Swartz from Lee County, Alabama were prepared in the same manner as *B.multifidum*. Specimens were viewed with a Zeiss DSM 940 at 5,10, or 15 kV. Qualitative element identification was performed using energy dispersive spectroscopy (Tracor Northern Micro Z II) coupled to the SEM.

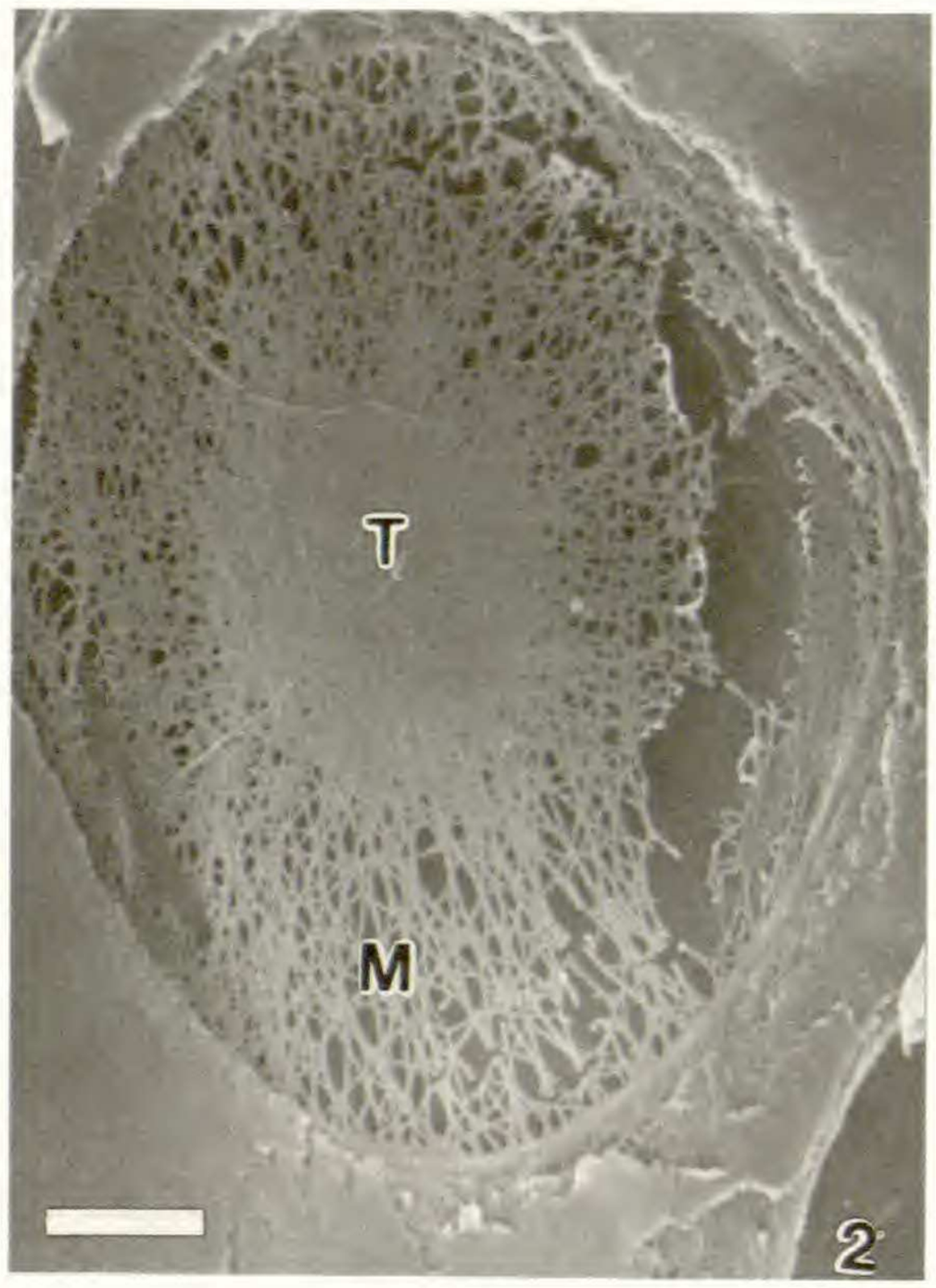
Results

Secondary xylem tracheids of *Botrychium multifidum* contain helical wall thickenings and intertracheid circular bordered pits (Fig. 1). Thickenings, as seen in longitudinal section, are uniform neither in height nor in distance between gyres, and thickenings are sometimes branched (Fig. 1). The pit membrane is almost always differentiated into a torus and margo (Fig. 2). Microfibrils of the pit membrane are loosely woven in the margo region, but tightly woven in the torus. Tearing of the pit membrane was sometimes evident in the margo (Fig. 2). Crystals were found in association with torus-bearing pit membranes of tracheids (Fig. 11), as well as in tracheid lumen (Figs. 1, 3). These crystals were not apparent at the light level. Crystals associated with these tracheids are styloids (Frey-Wyssling, 1981; Carlquist, 1988); they are rectangular columnar with pyramidal ends. Intact crystals have columns that are four-sided and are smooth-surfaced. The pyramidal crystal ends consist of four equilateral triangles, although wedge-shaped ends also were observed (Fig. 4). Crystal ends, when visible, typically appeared to be rough (Fig. 3), although some crystals with smooth ends were observed (Fig. 4). Crystals ranged in size from 4.3 to 12 μm in length, and 1.14 to 2.4 μm in width (N = 12). The mean crystal length is 7.27 μm, mean width is 1.55 μm, and mean ration of width-to-length is 1: 4.7. The crystals were not always regular in shape and sometimes appeared to have their growth modified by the presence of a helical wall thickening (Fig. 3). By energy dispersive spectroscopy (EDS), these crystals were found to be composed of a calcium compound, most likely calcium oxalate (Fig. 13).

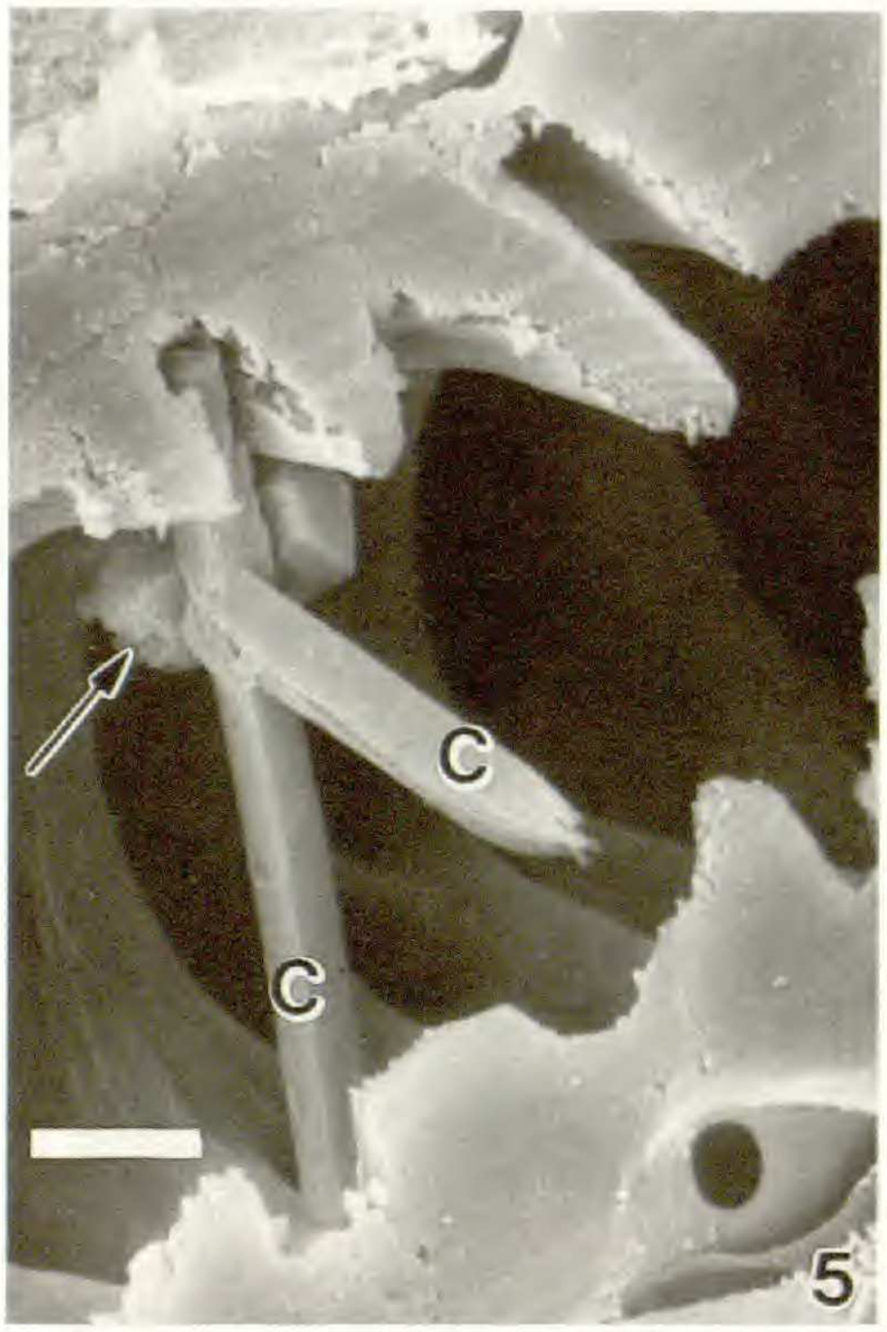
Although single isolated crystals were most commonly found, joined double crystals with U-shaped conjoined end were also observed (Fig. 4). Crystals in groups of two or more were also encountered and had either parallel or perpendicular orientation to each other (Figs. 1,5). Crystals were found in various positions within the tracheary lumen. They were located between either the helical thickenings of the wall material, laying flat on the inner cell wall, or projecting out from the pit membrane (Fig. 1).

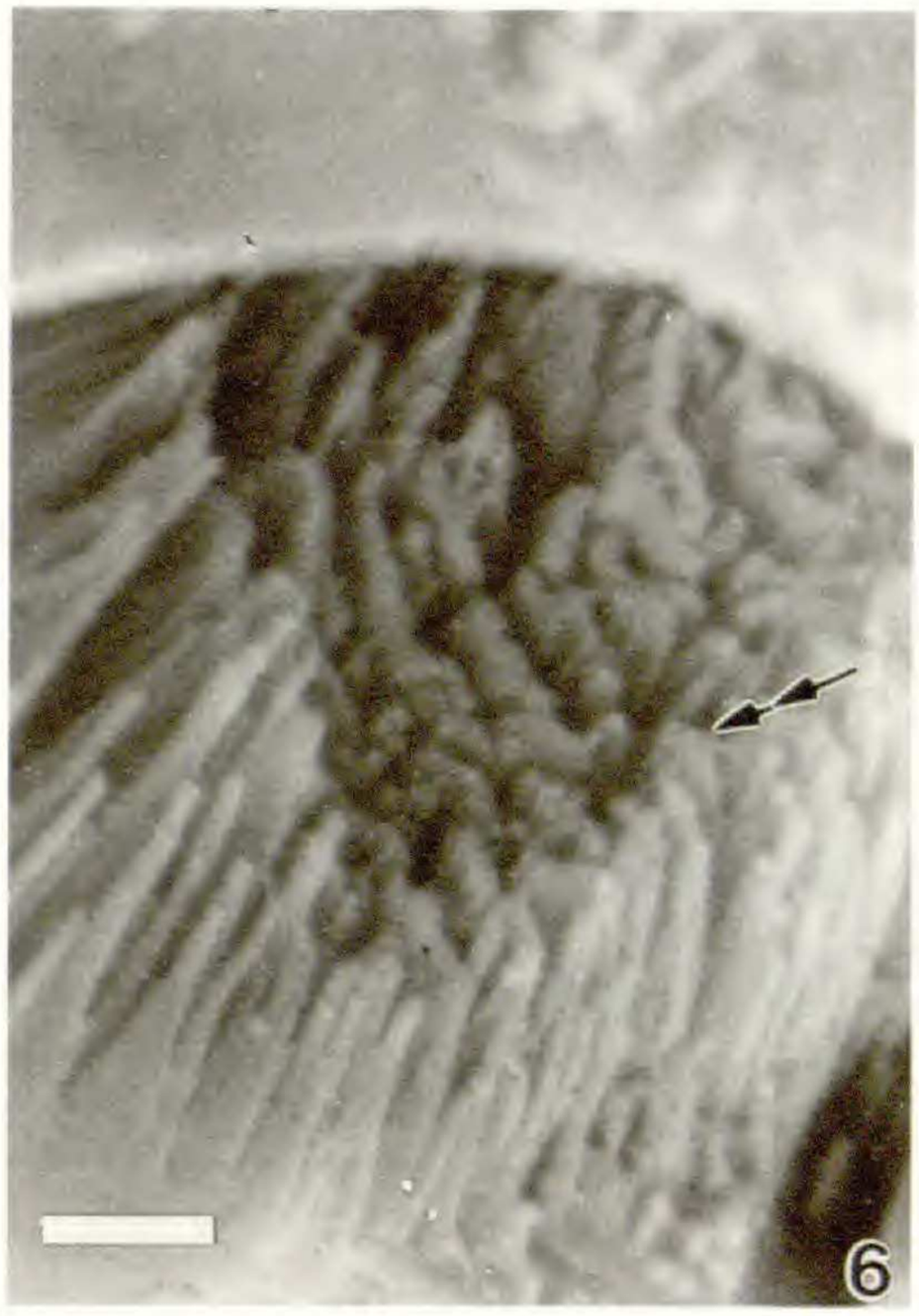
It was clear from some specimens that the crystals were composite structures (Figs. 4, 6–8). In some instances the subunits resembled raphides (Figs. 6,7),

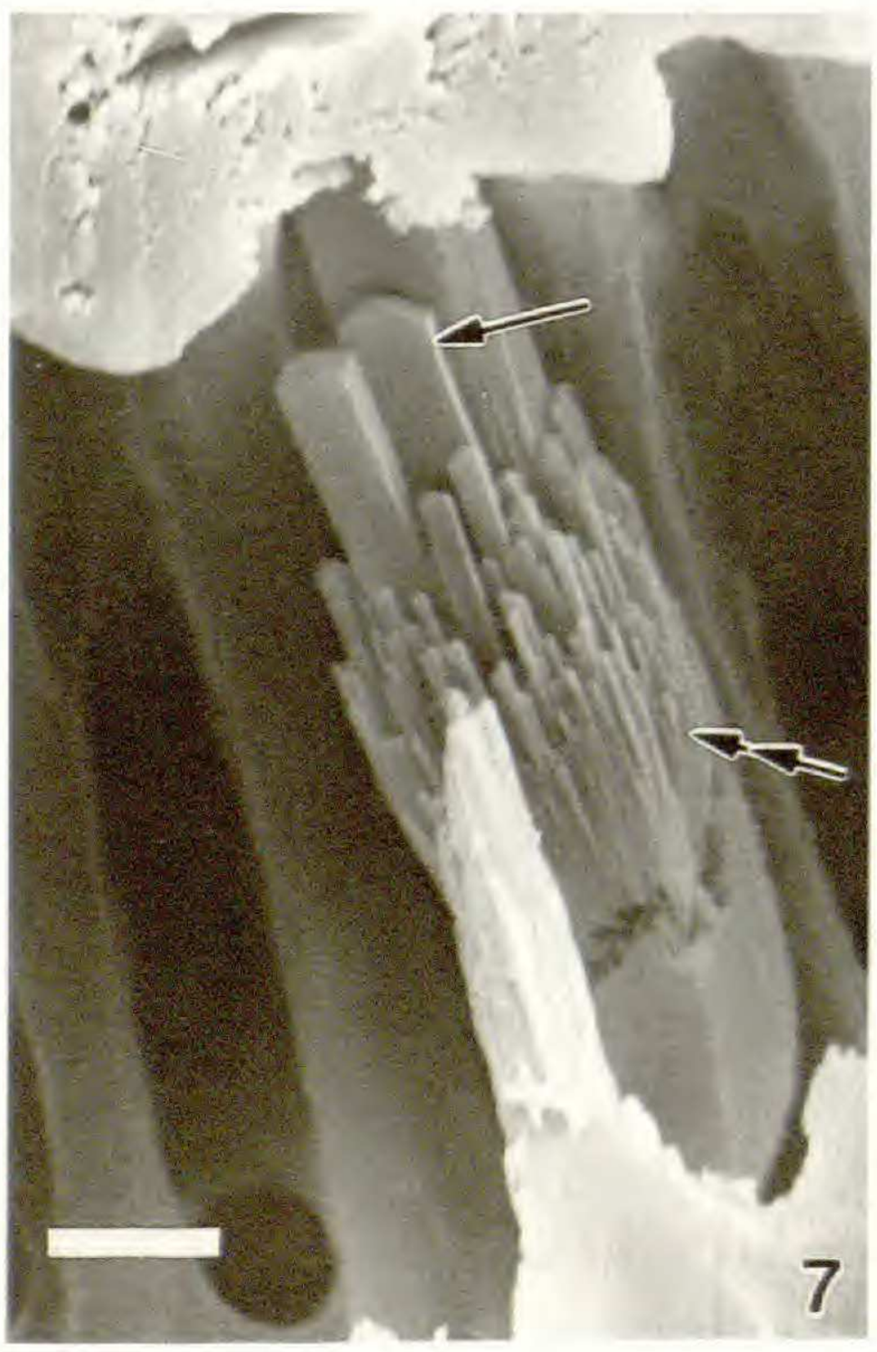


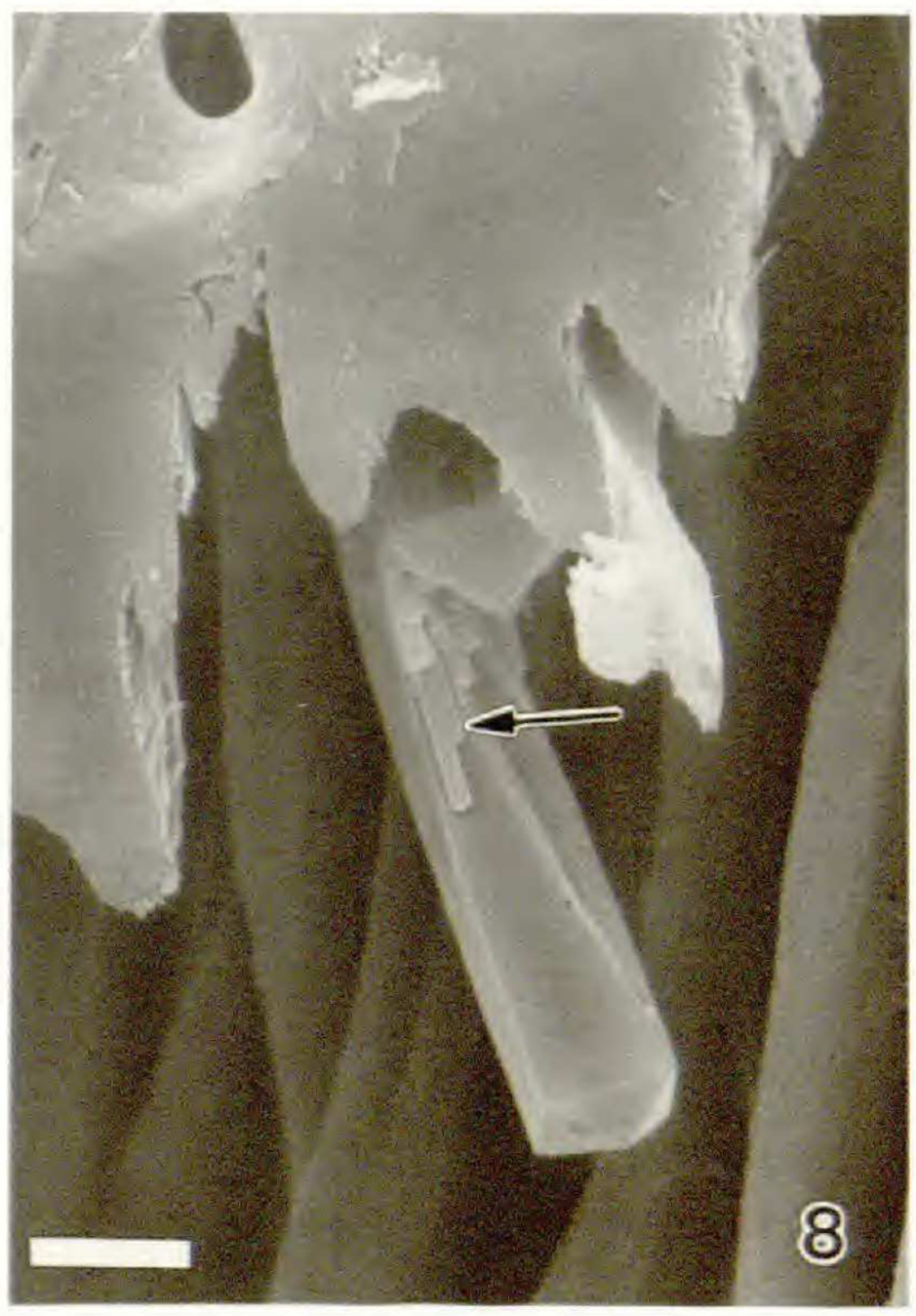


FIGS. 1–4. SEM micrographs of intertracheary pit membranes and crystals. 1) Tracheary lumen with helical wall thickenings (W), crystals (arrow), pit aperture (A), and circular bordered pit membrane (P); scale bar = 5 μm. 2) Intertracheary pit membrane with torus (T) and margo (M). The pit border was removed when the wood was split during preparation; scale bar = 2 μm. 3) Crystal entering a pit aperture (A). Note how the crystal appears to have grown around the helical thickening to the right (double arrow). R = rough end of crystal; scale bar = 2 μm. 4) Double crystal joined at one end (arrow); scale bar = 2 μm.









FIGS. 5–8. SEM micrographs of crystals in which crystals reveal their subunit composition. 5) Multiple crystals with parallel orientation and perpendicular orientation. Note subunits in broken crystal (arrow); scale bar = 5 μm. 6) End view of composite crystal formed by smaller raphide shaped crystals (arrow); scale bar = 500 nm. 7) Composite crystal with styloid (arrow) and raphide crystal (double arrow) shaped subunits; scale bar = 2 μm. 8) Composite crystal with styloid crystal subunits (arrow); scale bar = 2 μm.

whereas in others they resembled small styloid crystals that were fused to form one large crystal (Figs. 7, 8). Both types of subunits appear to integrate into one another (Fig. 7). One shattered example had a hollow center (Fig. 9).

Crystals were observed to traverse the pit aperture (Figs. 3, 10) and contact the pit membrane (Figs. 11, 12). These did not appear to penetrate the pit membrane, but we are uncertain of this point due to the poor preservation of the pit membranes in our samples. Fig. 12 demonstrates a unique occurrence in which a pit membrane is approached by a crystal from either side. Due to the position of the crystal relative to the pit membrane, we were unable to confirm the presence of a torus in each crystal-associated pit membrane; however a torus was present in the samples that exhibited a crystal behind the pit membrane (fig. 11).

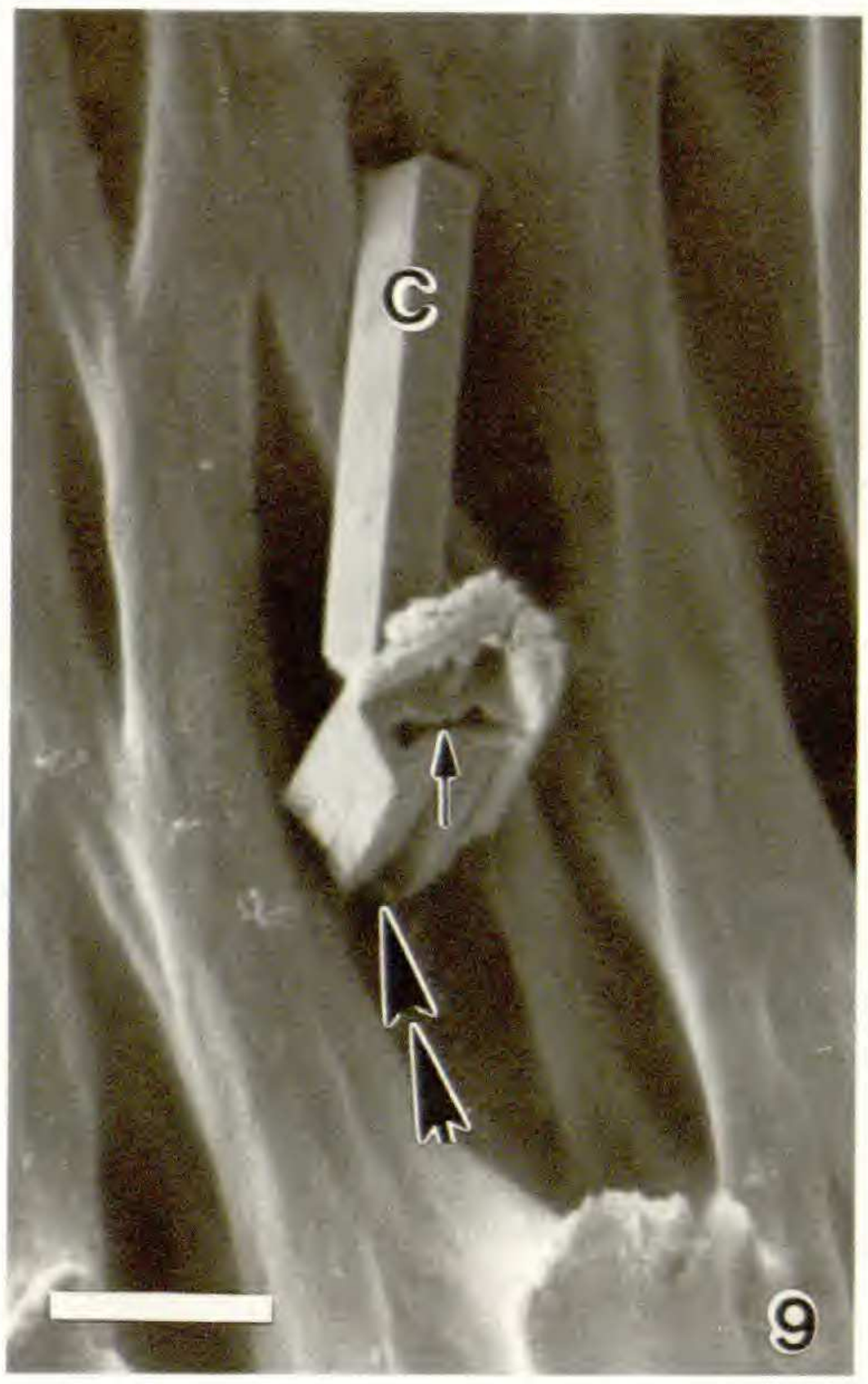
No noticeable chamber or crystal sheath was ever observed in association with a crystal. No evidence of a surrounding membrane was discovered, although the ends of the crystals often were rough (Fig. 3). Efforts to examine crystals with TEM to determine the presence or absence of a chamber or crystal sheath were not successful. Crystals were not observed in either *Botrychium dissectum* or *B. virginianum*.

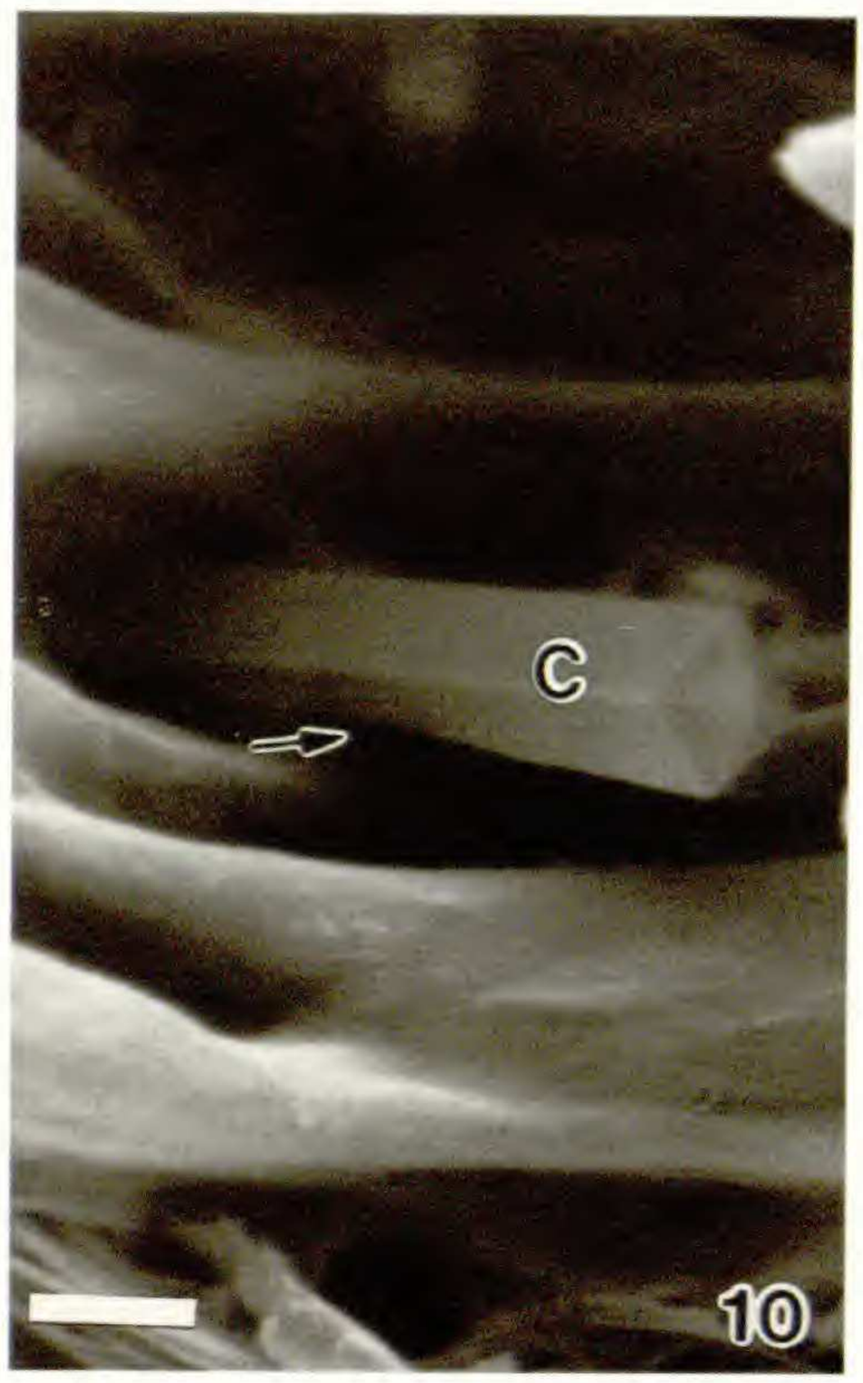
Discussion

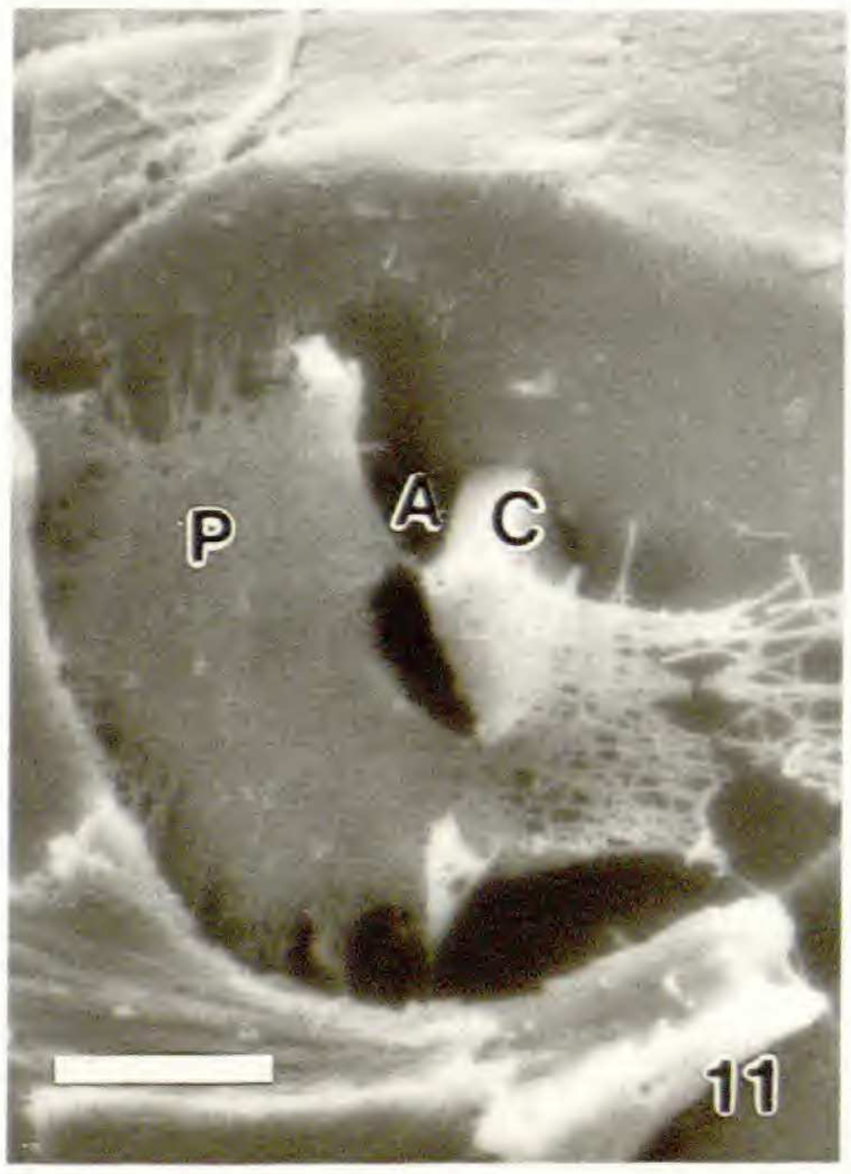
Three major systems of mineralization occur in plants. These include silicification, calcium carbonate crystallization, and calcium oxalate crystallization (Grimson et al., 1982). Calcium oxalate crystals, either in the monohydrate or polyhydrate state, are the most common mineral deposits (Webb and Arnott, 1982). EDS evidence indicates that our crystals contain calcium. The bipyramidal shape of the crystals' ends, and the rectangular columns, suggest that they are crystals of calcium oxalate in the polyhydrate form (Frey-Wyssling, 1981). Usually, acid solubility tests are used to confirm crystal composition in plants (Webb and Arnott, 1982). In addition, the oxalate nature of a calcium crystal can be tested with cupric acetate and ferric sulphate (Deshpande and Vishwakarma, 1992). However, due to the small size and sparse number of crystals found in *Botrychium multifidum,* these tests were not performed.

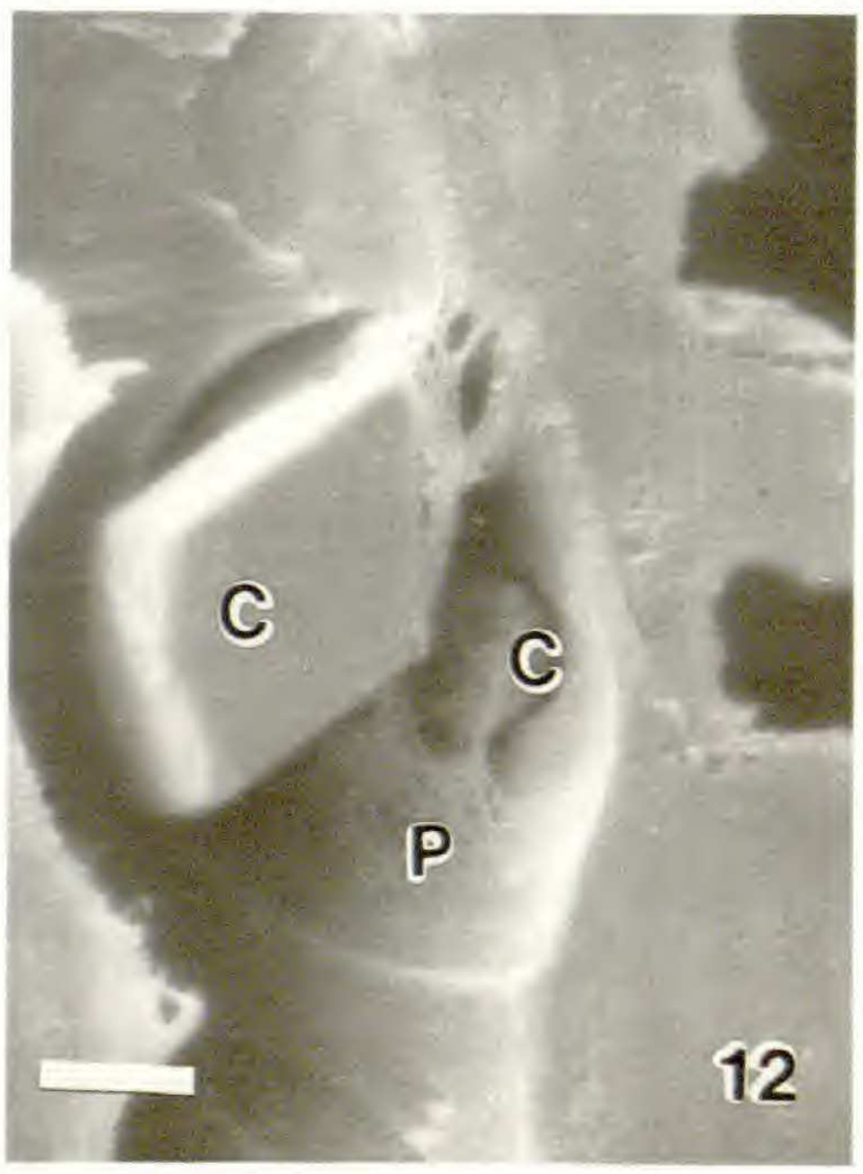
The location of crystals in tracheid lumens in unusual. Crystals in wood are most frequently found in ray or axial parenchyma cells (Chattaway, 1955, 1956), although they may also be found in septate fibers, vessel tyloses, and even in vascular cambia (Deshpande and Vishwakarma, 1992). In *Polyalthia,* vessels contained a crystalline mass (Scurfield and Mitchell, 1973). In the current study, crystals were isolated within the tracheary lumen, and there was no evidence suggesting attachment to cell walls. Some crystals appeared to be touching, but were not attached to, the pit membrane. Crystals also were found that had no apparent association with a pit membrane. Therefore, it appeared that crystal formation was not directly related to pit membranes.

Crystals in plants often are formed in membrane-bound compartments within the vacuole (Arnott and Pautard, 1970; Franceschi, 1984; Webb *et al.*, 1995). As proposed by Arnott and Pautard (1970), the cell membrane may control



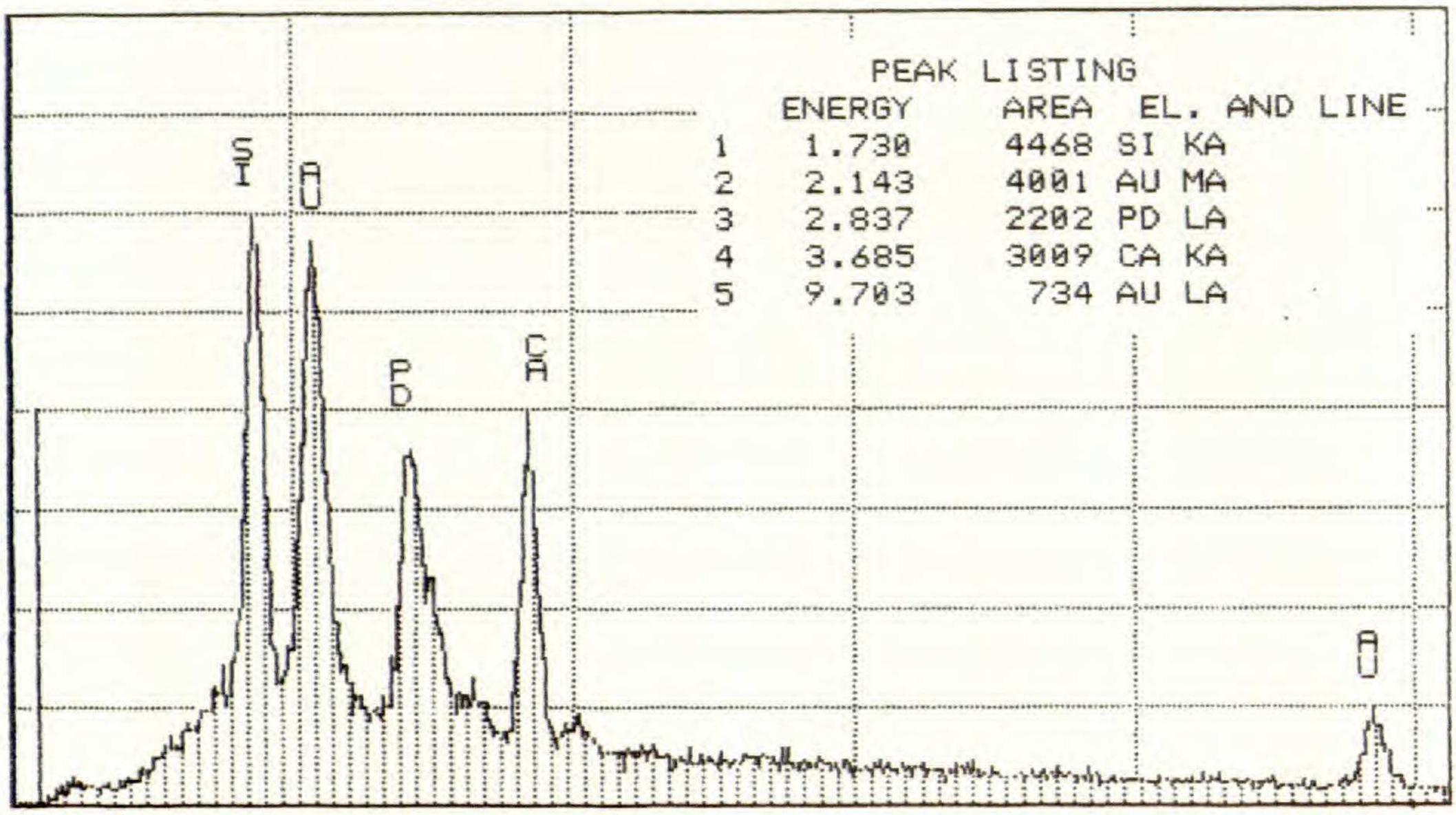






FIGS. 9–12. SEM micrographs of crystals in association with pit area and pit membranes. 9) Fractured crystal with hollow center (arrow). Note the aperture behind the crystal (double arrow); scale bar = 5 μm. 10) Crystal entering a pit aperture. Note shaping of the crystal around helical thickening (arrow); scale bar = 2 μm. 11) Crystal behind pit membrane; scale bar = 2 μm. 12) Pit membrane associated with crystals from contiguous tracheids; A = aperture; C = crystal; P = pit membrane; scale bar = 2 μm.

A. Experimental



B. Control

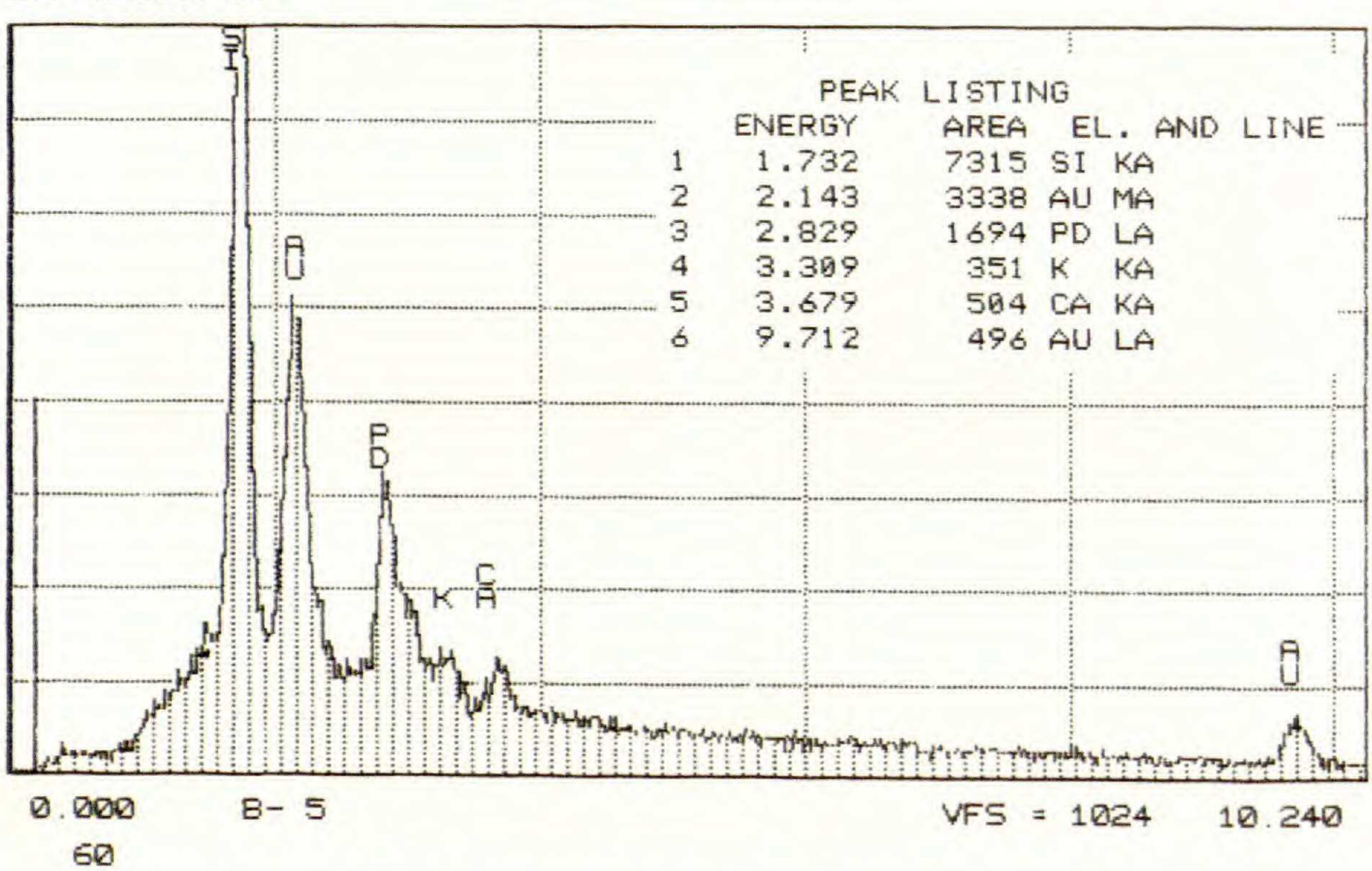


FIG. 13. EDS of tracheid. A. Spectral tracing of crystal within a tracheid. The calcium component of the spectrum is conspicuous and is indicated by the peak labeled CA. B. EDS of tracheid without crystal. Silicon, palladium, and gold are present as background elements (q.v. see preparation procedures in Materials and Methods). Only a small calcium peak is present. The latter probably represents calcium in the middle lamella. AU = gold; CA = calcium; PD = palladium; SI = silicon.

both shape and growth of crystals. There was no direct evidence that crystals of *B. multifidum* were once enclosed in a membrane, but Scurfield and Mitchell (1973) suggest that a rough area on a crystal is indicative of the adhering remnants of membrane. In crystals of *B. multifidum* only the membrane's impression on a crystal would be evident because living portions of the tracheid has undergone autolysis and no membrane remains. If the vacuole with its membrane-covered crystal pressed against either the cell wall or a cell wall thickening as a crystal formed, this contact could explain the shape of these crystals.

Water flow though the xylem could also deposit crystals (no longer enclosed by cytoplasm) randomly throughout a tracheid, including on top of a pit membrane or between wall thickenings. Due to erosion, water flow might also change crystal shape.

Another aspect of crystal development in plant cells in isolation of a crystal by wall material or a suberized sheath after the crystal has formed within a vacuole. This process would, in essence, externalize the crystal (Frank and Jensen, 1970). In *Agave* (Agavaceae), crystals are produced in such extraplasmic compartments (Wattendorff, 1976a, b). Wattendorff (1976b) found that all styloid idioblast of *Agave,* where they did not touch the wall, were surrounded by a suberized sheath. Although crystals of *B. multifidum* are styloids, they appear neither to be associated with a sheath of any sort nor to be isolated by cell wall material.

The reason a cell forms a crystal is not well understood. Crystal formation may represent a crystallization of waste material or storage of minerals (Deshpande and Vishwakarma, 1992). Crystal formation also may be associated with ionic balance, and therefore, the formation of a crystal could be a form of osmoregulation (Franceschi and Horner, 1980). Franceschi and Horner (1979) correlated the amount of calcium in the growth medium and the number of crystals formed in *Psychotria* L. (Rubiaceae) callus. Lane (1994) has suggested that calcium oxalate crystals may promote the polymerization of lignin which of course, would be occurring in the developing tracheids of B. multifidum.

It is evident at times that crystal formation in plants is under genetic control (Frey-Wyssling, 1981; Webb, 1999); however, genetic control of the formation of all crystals has not been proven. The cell in which a crystal is produced undergoes many changes at macro, micro, and ultrastructural levels, as well as, changes in cell chemistry. These changes, documented in other plant taxa during crystal formation, make it unlikely that crystal formation could be simply the result of precipitation or crystallization (Franceschi and Horner, 1980), although crystal formation may represent crystallization of waste material or storage of minerals (Deshpande and Vishwakarma, 1992). Deshpande and Vishwakarma (1992) also identified seasonal fluctuation in crystal formation after the cessation of cambial activity. Gourley and Grime (1994) described crystals that were more commonly found in the late wood of *Acacia* Mill.(Fabaceae). The availability of water was also determined to be a factor in crystal formation (Gourley and Grime, 1994).

It was impossible to determine for certain whether crystals in the tracheids

of *B. multifidum* formed before or after cell death. Perhaps due to greater water flow resistance occurring at the pit membrane, there would have been a greater chance for calcium precipitation in the pit area rather than in the tracheary lumen. If this were the case, crystals could at the pit membrane form after the death of a tracheid. However, the rough ends observed on some crystals suggest they may have been enclosed at one time by a membrane. Additionally, the crystals appear to conform to the shape of the pit aperture or cell wall thickenings and do not appear to have been randomly distributed by water flow. Perhaps the best explanation of where these crystals develop is in membrane compartment within vacuoles of living tracheids. The enlargement of a crystal in a plane perpendicular to the cell's axis would result in its abutting a wall or pit membrane, thus influencing crystal shape. Crystals that elongated parallel to a cell's axis would not encounter these boundaries and would not be shaped by them. Crystals that were not pressed into a cell wall or pit membrane also would not develop this shaping and might settle between wall thickenings, or after cell death, move with the xylem water stream. However, the positions of crystals in Figures 3, 10, and 12 with respect to the pit membrane suggest that crystal position is not the result of water flow.

Crystal formation has also been associated with the products of fungal metabolism within the plant cells (Scurfield and Mitchell, 1973). In our study, no fungal hyphae were found near any of the crystals. Therefore, this possibility in *B. multifidum* seems unlikely.

If crystal manufacture is under genetic control, what advantage does the cell gain from its production? This is an especially intriguing question with regards to *Botrychium* as crystal production would be occurring in cells about to die. Lane (1994) has suggested that calcium oxalate crystals play a role in lignin polymerization and perhaps this may be true in these lignified tracheids. However, the lack of crystals in other *Botrychium* species indicates that this would be true only under certain environmental conditions. As previously mentioned, some authors believe that crystals represent the storage of calcium that could be either reserve calcium or waste calcium (Deshpande and Vishwakarma, 1992; Webb, 1999). Storage of needed calcium in a near-death cell would be unlikely. However, these crystals may have been produced by a cell for ionic balance or osmoregulation. Ionic balance and osmoregulation are critical for immature cells (Franceschi and Horner, 1980). If a plant were growing on soil with high nitrate levels, assimilation of this compound would increase cell pH, and oxalic acid might be produced to counter this effect. The oxalate anion could then react with calcium to form a crystal that would remove the excess anion from cell sap (Franceschi and Horner, 1980).

Another explanation could be protection from herbivores, although crystal production in a leaf cell would be more plausible for defense purposes. The crystals in *B. multifidum* are too small and too few in number for this type of protection. Fire protection was listed as an explanation by Gourley and Grime (1994) for crystals in *Acacia*, but again this is unlikely for a rhizome.

Based on our data the best explanation for crystal formation in the xylem of *B. multifidum* is that crystals are the result of excess calcium precipitation,

which could represent either waste, storage, or osmoregulation in the plant. Because the crystals are located in dead cells, active resolubilization by a cell would be unlikely; however, if crystals were dissolved by water flow in the xylem, their minerals could be carried in the transpiration stream. Deshpande and Vishwakarma (1992) have suggested that formation of calcium crystals may be a reversible process in some tissues. Therefore, these crystals do not necessarily represent a calcium loss for the plant.

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